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# **In Silico Modeling of Olfactory Receptor 4M1 and Biochemical Characterization of Novel Ligands in Tauopathy Attenuation**

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Keywords:	traumatic brain injury, olfactory receptor, G protein-coupled receptors, in silico screening, tau phosphorylation, rodent model of mild traumatic brain injury

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**In Silico Modeling of Olfactory Receptor 4M1 and Biochemical Characterization of Novel Ligands in Tauopathy Attenuation**

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Running title: OR4M1 Ligands Attenuate Tauopathy

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**8 text figures & tables**

**Keywords**

- traumatic brain injury
- olfactory receptor
- G protein-coupled receptors
- in silico screening
- tau phosphorylation
- rodent model of mild traumatic brain injury

## Abstract

Traumatic brain injury (TBI) is a risk factor for several neurodegenerative disorders. We previously reported that abnormal tau processing, a canonical feature in Alzheimer's disease and other tauopathies, may be traced back to abnormal down-regulation of certain ubiquitous olfactory receptors (ORs) in the brain, eg., the OR4M1 subtype, in subjects with a history of TBI. The objective of this study was to investigate the role of OR in TBI mediated tau pathology and the feasibility of using OR ligands as a novel therapeutic intervention. We built a 3-D OR model and used *in silico* screening of potential novel ligands from commercial drug libraries able to functionally activate OR signaling and eventually attenuate the formation of abnormal tau. Excitingly we found that *in vitro* activation of OR4M1 with the commercially available ZINC library compound 10915775 led to a significant attenuation of abnormal tau phosphorylation in embryonic cortico-hippocampal neuronal cultures derived from NSE-OR4M1 transgenic mice, possibly through modulation of the JNK signaling pathway. The attenuation of abnormal tau phosphorylation was rather selective since ZINC10915775 significantly decreased tau phosphorylation on tau Ser202/T205 (AT8 epitope) and tau Thr212/Ser214 (AT100 epitope), but not on tau Ser396/404 (PHF-1 epitope). Moreover, no response of ZINC10915775 was found in control hippocampal neuronal cultures derived from wild type littermates. Our *in silico* model provides novel means to pharmacologically modulate select ubiquitously expressed ORs in the brain through high affinity ligand activation to prevent and eventually to treat TBI-induced down regulation of ORs and subsequent cascade of tau pathology.

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Traumatic brain injury (TBI) is an acquired injury caused by a sudden trauma to the head which disrupts normal brain functioning, leading to either transient or chronic impairments in physical, cognitive, emotional, and/or behavioral functions. In the civilian population, TBI is typically associated with direct, closed impact mechanical trauma to the brain due to falls, motor vehicle accidents, sports, *etc.* (Elder & Cristian 2009b). In contrast, TBI among military personnel, particularly among Veterans returning from the Persian Gulf region as part of service in Operation Iraqi Freedom (OIF) or Operation Enduring Freedom (OEF), is primarily due to exposure to blast pressure waves stemming from blast-producing weaponry, such as improvised explosive devices (IEDs) (Elder & Cristian 2009a).

We previously reported decreased expression of select olfactory receptors in subjects with TBI history (Zhao et al. 2013a) and found an association between abnormal tau formation and decreased expression of certain ubiquitously expresses ORs in the brain and in circulating mononuclear cells. ORs are members of the class A rhodopsin-like family of G protein-coupled receptors (GPCRs). While ORs have been largely investigated for their role in olfaction, little is known about their role in the brain. For example, it is well known that specific odorants, when bound to select ORs in the olfactory mucosa, may transduce the sensation through OR-type G proteins consistent with the general concept that GPCRs, like olfactory ORs are membrane-bound proteins that are responsible for cell-environment communications (Horn et al. 1998). Moreover, recent evidence suggests conditions associated with inflammation, obesity, hypertension as well as mood disorders such as anxiety and depression are also associated with altered GPCRs (Wilson & Bergsma 2000), making them among the most important targets for pharmacological intervention (Rattner, Sun, & Nathans 1999; Sautel & Milligan 2000; Schoneberg, Schulz, & Gudermann 2002). Unfortunately, GPCRs, like other membrane-embedded proteins, have characteristics that make their 3-D structure difficult to determine experimentally. While the structures of several GPCRs have been determined recently, no experimental structures of ubiquitously expressed ORs are currently available.

The current study is designed to identify ligands for the ubiquitously expressed OR4M1 via in silico screening using a homologous model of OR4M1 and to discover potential novel receptor ligands able to be further develop as future new treatment of abnormal tau processing in TBI and other neurodegenerative disorders.

## Materials and Methods

### *Homology Modeling and Binding Site Identification*

A homology model of OR4M1 was built using MODELLER version 9.12 (Eswar et al. 2003) using bovine rhodopsin as a template. Because the sequence similarity between OR4M1 and bovine rhodopsin is below 25% identity, the alignment of the two sequences was built using profiles of homologous olfactory receptors and other GPCRs. The profiles were matched to each other using the SALIGN routine of program MODELLER (Marti-Renom, Madhusudhan, & Sali 2004). The alignment was manually edited using predictions of transmembrane helices for OR4M1. The quality of the alignment was evaluated by mapping predicted olfactory receptor binding site residues (Man, Gilad, & Lancet 2004a) onto the model. A divergent loop (Ala175-Pro175) was remodeled using the loop modeling routine of MODELLER (Fiser, Do, & Sali 2000). A total of 100,000 conformations for the loop were generated and the best scoring one, based on the DOPE potential (Shen & Sali 2006), was selected as the final model. The ligand-binding pocket in the final model was defined by the known OR binding site residues and by the SiteHound binding site identification program (Gherssi & Sanchez 2009b).

### *Docking, Scoring and Selection*

The pocket identified in the OR4M1 model was used as a target for virtual screening of small molecular compounds. A set of ~ five million lead-like compounds (Teague et al. 1999) derived from the ZINC library of commercially available compounds (Irwin & Shoichet 2005b) was docked into the binding site using program DOCK version 6.5 (Moustakas et al. 2006). Lead-like compounds were selected to facilitate optimization of validated hits. Compounds for experimental testing were selected based on docking score and visual inspection of binding site complementarity and hydrogen bonding. Clustering of compounds based on chemical fingerprint similarity was carried out using single-linkage clustering and using a Tanimoto coefficient of 0.7 as the cutoff. Chemical fingerprint similarity was computed using the JChem software (ChemAxon).

### *Experimental Animals*

Rat neuron-specific enolase (NSE) promoter plasmid containing human OR4M1 was constructed by inserting ~1kb cDNA fragment with the entire coding region of hOR4M1 (NM\_001005500.1, OriGene Technologies, Inc. Rockville, MD) in the NotI site of the plasmid vector. A cassette of ~6 kb SalI fragment containing NSE promoter and hPGC-1 $\alpha$  was gel purified and microinjected into one-cell mouse egg (C57BL6 x SJL) as described previously (Kelley et al. 1999; Qin et al. 2006). TgOR4M1 founders were identified by PCR-based genotyping. All animals were maintained on a 12:12-h light/dark cycle with lights on at 07:00 h in a temperature-controlled (20  $\pm$  2°C) vivarium, and all procedures were approved by the MSSM IACUC.

Mild TBI (mTBI) rodent models were generated by blast exposure, as previously described (Chavko, Prusaczyk, & McCarron 2006). Briefly, adult male Long Evans hooded rats (250–350g; 10–12 weeks of age) were used as subjects. Rats were exposed to overpressure injury using the Walter Reed Army Institute of Research (WRAIR) shock tube, which simulates the effects of air blast exposure under experimental conditions (Elder et al. 2012). Blood samples were withdrawn through the saphenous vein 1, 3, 6, and 12 months following the blast. At the end of the study, the cortex, hippocampus and cerebellum were collected and stored at -80°C until further analysis.

### *Primary neuron preparation, treatment and cAMP assay*

Embryonic day 15 cortico-hippocampal neuronal cultures were prepared from TgOR4M1 mice. Neurons were seeded onto poly-D-lysine-coated 12-well plates at  $5 \times 10^5$  cells per well and cultured in Neurobasal medium supplemented with 2% B27, 0.5mM L-glutamine, and 1% penicillin-streptomycin (all from Life Technologies). On Day 5 of the culture, primary cortico-hippocampal neuron cultures were pretreated with 3-isobutyl-1-methylxanthine (IBMX, from Sigma-Aldrich), an inhibitor of cAMP phosphodiesterase, for 10 min followed by ligand treatment (10 $\mu$ M, all from Enamine) for 10 min. cAMP assay was performed using a colorimetric cAMP ELISA assay kit (Cell Biolabs) according to the manufacturer's instructions.

### *RNA extraction, cDNA synthesis and quantitative PCR*

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Rat blood RNA was extracted using the Ribopure RNA Purification kit (Life Technologies). One µg of total RNA was reverse transcribed using the SuperScript III first-strand synthesis kit (Life Technologies). Quantitative PCR (qPCR) was performed in four replicates in the 7900HT Fast Real-time PCR system (Applied Biosystems) using the Power SYBR Green PCR Master Mix (Applied Biosystems). Primers used in qPCR assay are summarized in Table I. Fold changes were calculated using the  $2^{-\Delta\Delta C_t}$  method as previously described (Zhao et al. 2013b).

**Assessment of tau phosphorylation**

Twenty µg protein lysate of either primary neuron or rat hippocampus samples were used for SDS-PAGE protein separation. Separated proteins were transferred onto nitrocellulose membrane, and western blotting was performed using 5% milk in Tris-buffered saline solution for blocking nonspecific binding sites and antibody dilutions. Primary antibodies against tau proteins included mouse monoclonal antibodies as follows (location of epitopes refers to the longest tau isoform of 441 amino acid residues): AT8 (pSer202/205), AT100 (pThr212/Ser214) and PHF-1 (pSer396/404).

For assessment of active JNK, anti - phospho-JNK (Cell Signaling) was used as probe and β-actin signaling (Santa Cruz Biotechnology) was used controlled for samples loading.

**Statistics**

In these studies, all values are expressed as mean and standard error of the mean (SEM). Differences between means were analyzed using one-way ANOVA or two-tailed student t-test. In all analyses, the null hypothesis was rejected at the 0.05 level. All statistical analyses were performed using the Prism Stat program (GraphPad Software, Inc.).

## Results

### *Modeling of OR4M1*

A model of the 3-D structure of olfactory receptor OR4M1 was built by comparative modeling based on the crystal structure of bovine rhodopsin (Okada et al. 2004). Most of the predicted binding site residues are spatially close in the model suggesting that the alignment is reliable. The alignment shows that the region between Ala 142 and Pro 175 of OR4M1 is highly divergent from the equivalent region in rhodopsin, hence this region was further refined using via loop modeling. In the final model most of the predicted binding site residues from Man *et al.* (Man, Gilad, & Lancet 2004b) cluster into one region (Fig. I) which is consistent with a binding pocket identified in the model using program SiteHound (Gherzi & Sanchez 2009a).

### *Docking and in silico screening*

The pocket identified in the OR4M1 model was used as a target for virtual screening of a set of ~ five million lead-like commercially-available compounds (Irwin & Shoichet 2005a). The top 700 hits from the screening (ranked by docking score) were visually inspected, and 57 compounds, which showed good occupancy of the predicted binding pocket and at least two hydrogen bonds with the protein, were selected, shown as a space filling model (Fig. IIA) and with predicted binding pocket (red mesh) (Fig. IIB). The selected compounds were clustered based on chemical similarity. This resulted in 32 clusters of structurally similar compounds. Based on the availability of these compounds, we chose 25 commercially available compounds that could be further grouped into 16 clusters of structurally similar compounds (Table II) for experimental validation.

### *In vitro validation of candidate OR4M1 ligands*

In this study, embryonic cortico-hippocampal neuronal cultures from Tg NSE-OR4M1 mice were used as test candidates' compound to activate OR4M1. The intracellular signaling pathways activated by GPCR signaling include the cAMP/JNK pathway among others. Since OR4M1 belongs to the GPCR family, we treated TgOR4M1 neuronal cultures with individual ZINC ligands at 10  $\mu$ M concentration and assessed for OR4M1-mediated cellular induction of cAMP as an index of OR4M1 activation.

We found that among the 25 compounds tested, ZINC10915775 was particularly efficient in the activation of OR4M1 as indicated by increases in cellular cAMP contents in response to ligand treatment (24 hr) (Table II). These ring structures occupied the two wider regions in the OR4M1 pocket, while the linker occupied the narrow connecting region (Fig. III A, B).

Based on this evidence, we continued to test the dose-responsiveness of activation of OR4M1 by ZINC10915775 in E15 cortico-hippocampal cultures generated from OR4M1 transgenic mice. Using the cAMP assay, we found that the activation of OR4M1 by ZINC10915775 reached peak activity at 100 nM (Fig. IIIC), so we chose this concentration for further *in vitro* mechanistic studies.

### *OR4M1 ligand ZINC10915775 inhibited tau phosphorylation through inhibition of c-Jun N-terminal kinase (JNK) signaling*

Based on this evidence we continued to explore whether OR4M1 activation could influence abnormal tau processing. In this study cortico-hippocampal neuron cultures derived from either TgOR4M1 mice or WT littermates ZINC10915775 (100 nM for 12 hrs). We found that treatment of TgOR4M1 neurons with the ZINC10915775 compound significantly decreased tau phosphorylation on Ser202/T205 (AT8 epitope) and Thr212/Ser214 (AT100 epitope), but not on Ser396/404 (PHF-1 epitope) (Fig. IVA).

Interestingly, we also found that attenuation of abnormal tau phosphorylation in the TgOR4M1 cortico-hippocampal neuron cultures in response to ZINC10915775 treatment coincided with a significant decrease in the levels of phosphorylated JNK (Fig. IVA).

However, no detectable change in tau phosphorylation or JNK was detected in WT neuron cultures upon ZINC10915775 treatment (Fig. IVB), suggesting that ZINC10915775-mediated activation of OR4M1 could lead to decreased tau phosphorylation, possibly through the inhibition of the JNK signaling pathway.



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***Expression of OR and tau phosphorylation in a rat model of blast-induced TBI (bTBI)***

To examine whether the down-regulation of OR4M1 as well as the expression of three other ubiquitously expressed OR previously found decreased in subjects with histories of TBI (Zhao et al. 2013c) we explored the expression of the rodent OR orthologs in an *in vivo* rat model of bTBI for potential future *in silico* drug screening studies.

Excitingly, we found that similar to what was found in subjects with a history of TBI, the expression of Olr1612, Olr1671, Olr322 and Olr735 which are the rodent orthologs of the human, OR4M1 OR2J3, OR4D9 and OR4X1, respectively, showed a time-dependent decrease in PBMCs at 1, 3, 6, and 12 months in the same rat group post-bTBI compared to naïve control rats (Fig. V).



## Discussion

The functional limitations and costs related to traumatic brain injury (TBI) place a profound burden on individuals and their families in the United States military and civilian communities (Leibson et al. 2012). With improved medical care, TBI morbidity and hospitalizations have declined 20% and 50% since 1980, but have resulted in more long-term morbidity and disability (Thompson, McCormick, & Kagan 2006). Long term disability after TBI has been attributed to cognitive and neurological disorders. Given the association with cognitive and neurobehavioral changes, there is increasing speculation that TBI is associated with an increased risk of Alzheimer's disease and other tauopathies, including Lewy Body dementia (LBD), Frontotemporal dementia (FTD), among others. However, epidemiological studies linking TBI and ADRC have resulted in conflicting data. Some studies have reported an association between TBI and an increased risk for developing Alzheimer's disease and related conditions, including tau misfolding and abnormal processing on of  $\beta$ -amyloid (Barnes et al. 2014; Bazarian et al. 2009; Graves et al. 1990; Guo et al. 2000; Molgaard et al. 1990; Mortimer et al. 1991; Wang et al. 2012), but many others have found no such risk (Chandra et al. 1989; Himanen et al. 2006; Mehta et al. 1999; Millar et al. 2003; Williams et al. 1991).

The current study is based on our recent observation that abnormal tau processing in subjects with a history of TBI may be traced back to abnormal down-regulation of certain ORs, including OR4M1 and OR11H1, in the brain (Zhao *et al.*, 2013). In this study, we demonstrated that activation of OR4M1 with select ligands led to reduction of phosphorylation at Ser202/205 and Thr212/Ser214 of tau and found that the reduction of tau phosphorylation was associated with altered activation of the JNK pathway. Interestingly the physiological relevance of OR4M1 is consistent with the recent study demonstrating that controlled cortical impact TBI activates JNK and increases tau phosphorylation in a 3xTg mouse model of AD (Tran *et al.*, 2013).

To explore the cause-effect relationship between TBI and OR down-regulation, and TBI-type neuropathology, we used a bTBI rat model. Consistent with our observation in human TBI subjects (Zhao *et al.*, 2013), we found significantly lower contents of OR4M1, and other select ORs previously found in the blood of blast-injured rats compared to control rats over 12 months following TBI exposure.

Our studies have identified a novel ligand for OR4M1 TBI biomarker through *in silico* screening, and activation of OR4M1 by this ligand in primary neuronal cultures resulted in decreased tau phosphorylation through inhibition of the JNK signaling pathway. Taking these findings together, our hypothesis is that inactivation of certain ubiquitous ORs in response to conditions associated with TBI might be at the basis for potential abnormal tau phosphorylation possibly through mechanisms associated with the JNK pathway (Fig. VI). This evidence suggests a potential fine-tuning character of OR-mediated JNK regulation in response to conditions associated with TBI.

The sequence of early tau phosphorylation suggests that there are events prior to filament formation that are specific to particular phosphorylated tau epitopes, leading to conformational changes and cytopathological alterations. There is evidence that a healthy neuron develops immunoreactive punctate phospho-tau inclusions, primarily AT8 and AT100, which eventually become preNFTs which give rise to a filamentous intra-cellular inclusion. Eventually the neuron dies and an extra-neuronal NFT, or ghost tangle remains. Our evidence that the ZINC10915775 may attenuate AT8 and AT100, which are indexes of the early stages of NFT formation, through the activation of OR4M1, supports the hypothesis that certain ligands of ubiquitous OR expression could be developed to target the very early stages of TBI associated ADRC, such as tauopathy (Augustinack *et al.* 2002).

Our observation from the experimental bTBI model demonstrated, for the first time, a direct cause-and-effect relationship between long-term down regulation of select ORs and a neuropathological feature in TBI. This validates our hypothesis that down regulation of select ORs in PBMCs and in the brains of our human TBI study cohort may be caused by prior TBI exposure (Zhao *et al.*, 2013). Our 3-D *in silico* screening model provides an innovative means to identify novel compounds that can modulate tau phosphorylation through activation of ORs. Future studies will focus on structure-activity relationships (SAR) around the active compound and the optimization of an *in silico* screening system based on this

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information to identify compounds with higher potency and specificity as potential preventative and possibly therapeutic agents for TBI associated ADRC at early stages.

**Conflict Of Interest**

The authors declare no conflict of interest.

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Legend

Table I. List of primers used in qPCR assays.

Figure I. *In silico* 3-D model of OR4M1 and predicted binding pocket.

The predicted ligand binding residues from Man *et al.* (Man, Gilad, & Lancet 2004c) are shown in yellow. The predicted binding pocket identified by SiteHound (Ghera & Sanchez 2009c) is shown as a red mesh. Additional residues that contribute to the binding pocket, but are not identified in Man *et al.* are shown in green.

Figure II. Example of virtual screening hit.

In panel A, one of the top scoring compounds is shown in cyan as a space-filling model. Protein binding site side residues are colored as in Figure 1. In panel B, compatibility of the same top scoring compound is shown with predicted binding pocket (red mesh).

Table II. Experimental validation of OR4M1 ligands identified by *in silico* screening; two-tailed t-test  
\*P< 0.05 vs vehicle control

Figure III. Structure, docking of the two experimentally validated hits in the OR4M1 binding pocket and dose response assay.

In panel A, structure of ZINC10915775; in panel B, predicted binding position for ZINC10915775 compound in the OR4M1 binding pocket; in panel C, dose dependent activation of OR4M1 by ZINC10915775. In panel C, the statistical analysis for the sigmoidal curve was generated by Prism Stat program (GraphPad Software, Inc.); n=3-4 per culture condition.

Figure IV. OR4M1 ligand ZINC10915775 decreased tau phosphorylation and inhibited JNK signaling in primary neurons.

Embryonic day 15 cortico-hippocampal neuronal cultures from TgOR4M1 mice (A) and wild type mice (B) were treated with 100 nM ZINC10915775 for 1 hour and western blot analysis were performed to assess tau phosphorylation and JNK signaling. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, by two-tailed t-test; n=3-4 per culture condition.

Figure V. Long term effect of blast on the peripheral olfactory receptor levels in a blast-induced rodent model of TBI.

Blood was withdrawn at 1, 3, 6, and 12 months following the blast. Quantitative PCR was performed using ABI 7900HT Fast Real-Time PCR System using primers specific for (A) Olr1612; (B) Olr1671; (C) Olr322; (D) Olr735. \*p<0.05, n=4-5 rats per group by 2-tailed t-test.

Figure VI. Scheme of working hypothesis.

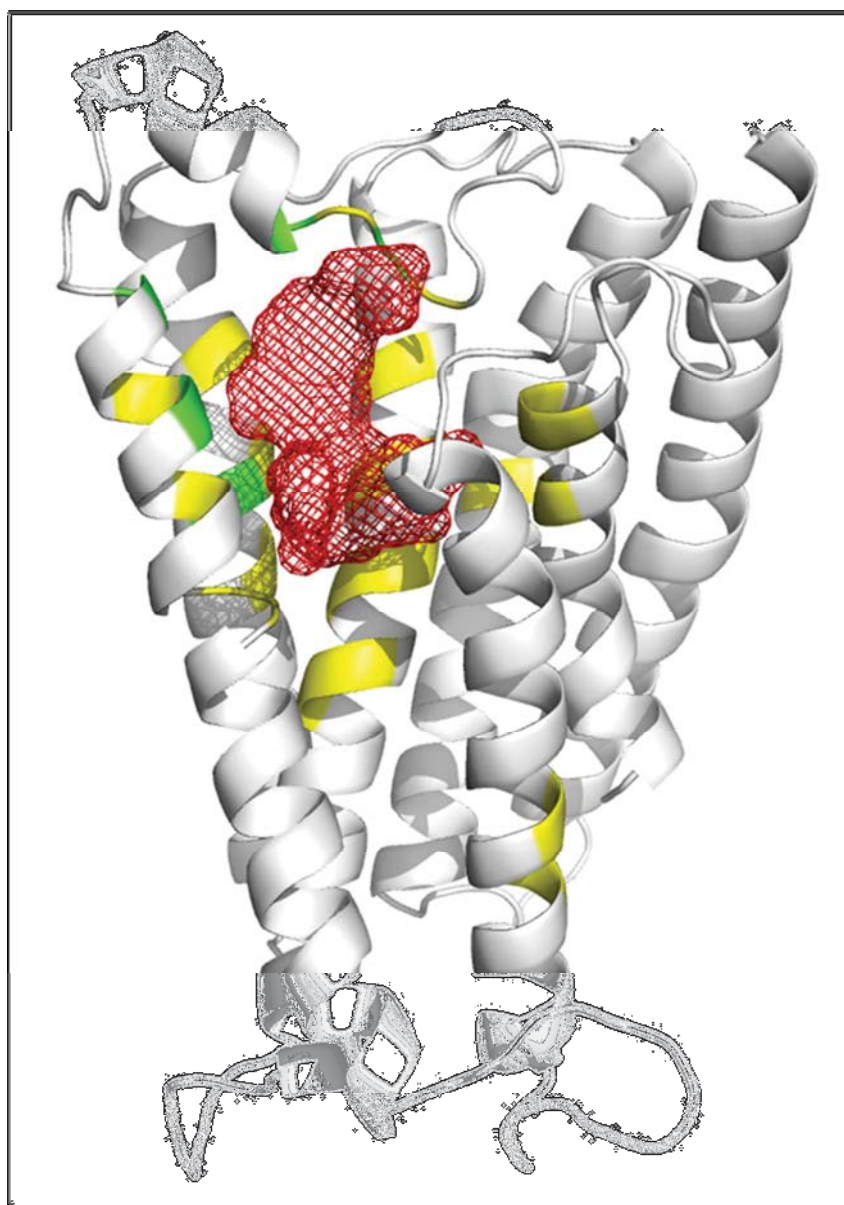


Figure I. In silico 3-D model of OR4M1 and predicted binding pocket. The predicted ligand binding residues from Man et al. (Man, Gilad, & Lancet 2004b) are shown in yellow. The predicted binding pocket identified by SiteHound (Gherzi & Sanchez 2009b) is shown as a red mesh. Additional residues that contribute to the binding pocket, but are not identified in Man et al. are shown in green.

117x171mm (148 x 143 DPI)

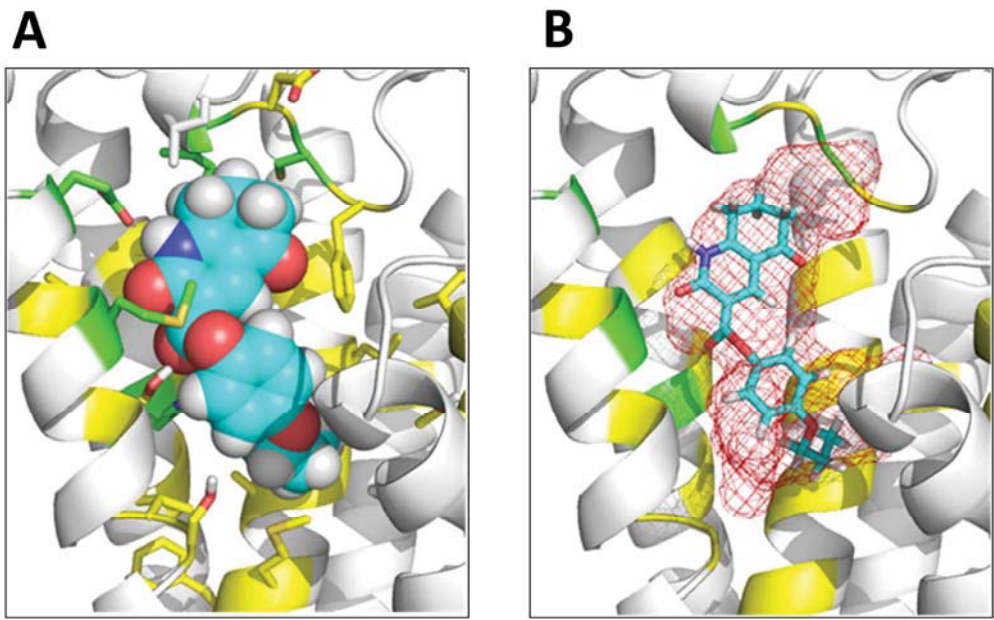


Figure II. Example of virtual screening hit.  
In panel A, one of the top scoring compounds is shown in cyan as a space-filling model. Protein binding site side residues are colored as in Figure 1. In panel B, compatibility of the same top scoring compound is shown with predicted binding pocket (red mesh).  
220x137mm (300 x 300 DPI)

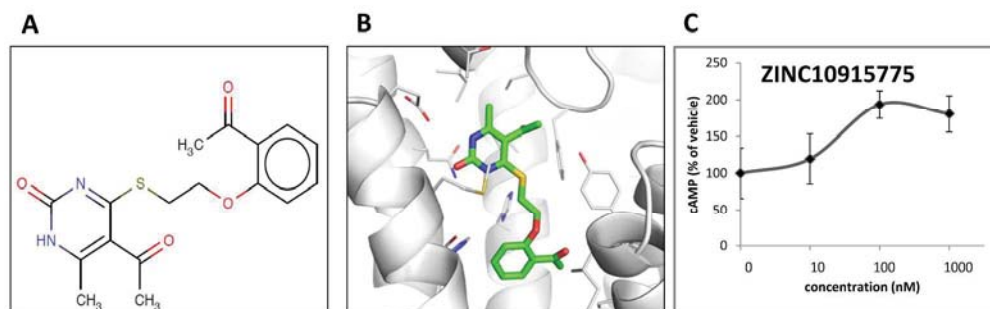


Figure III. Structure, docking of the two experimentally validated hits in the OR4M1 binding pocket and dose response assay.

In panel A, structure of ZINC10915775; in panel B, predicted binding position for ZINC10915775 compound in the OR4M1 binding pocket; in panel C, dose dependent activation of OR4M1 by ZINC10915775. In panel C, the statistical analysis for the sigmoidal curve was generated by Prism Stat program (GraphPad Software, Inc.); n=3-4 per culture condition.

246x76mm (300 x 300 DPI)

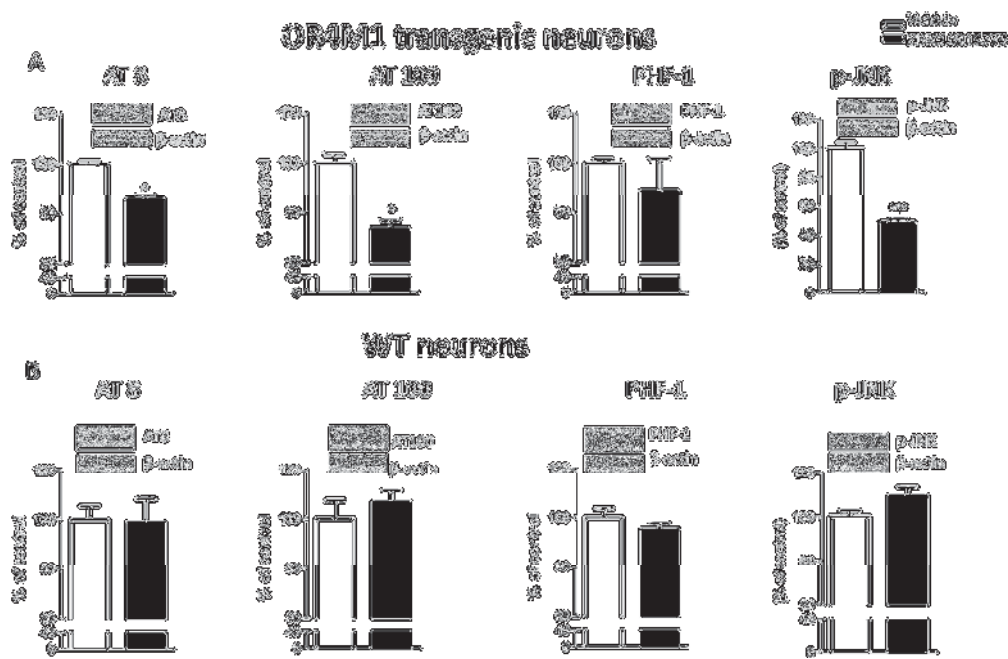


Figure IV. OR4M1 ligand ZINC10915775 decreased tau phosphorylation and inhibited JNK signaling in primary neurons. Embryonic day 15 cortico-hippocampal neuronal cultures from TgOR4M1 mice (A) and wild type mice (B) were treated with 100 nM ZINC10915775 for 1 hour and western blot analysis were performed to assess tau phosphorylation and JNK signaling. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , by two-tailed t-test;  $n = 3-4$  per culture condition.

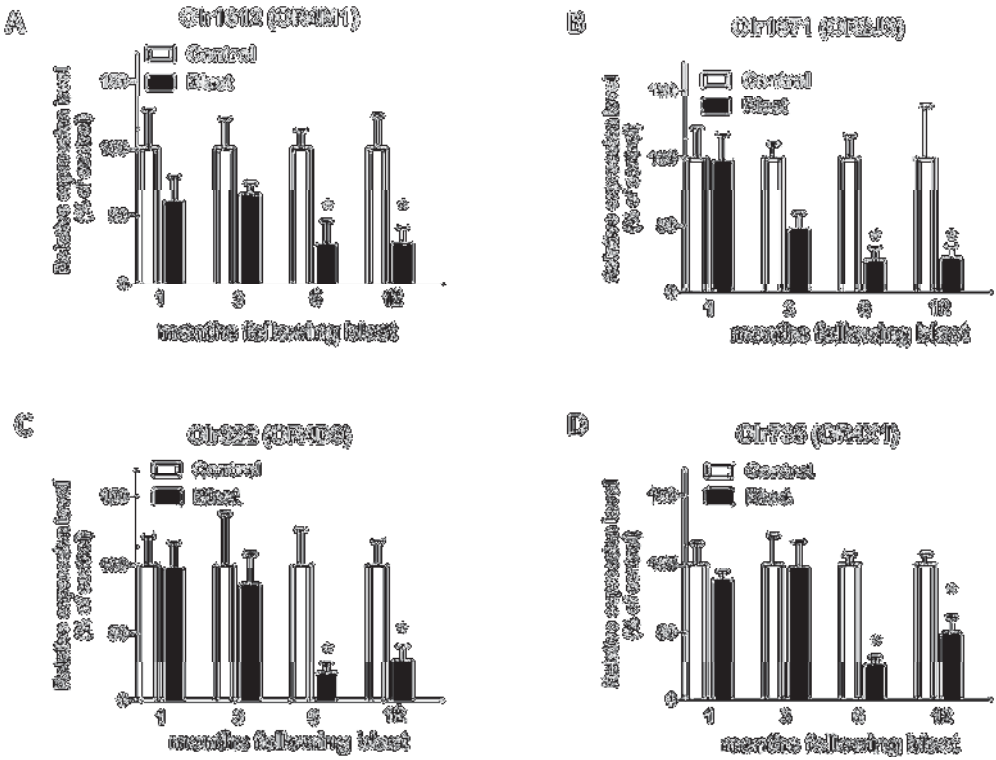


Figure V. Long term effect of blast on the peripheral olfactory receptor levels in a blast-induced rodent model of TBI. Blood was withdrawn at 1, 3, 6, and 12 months following the blast. Quantitative PCR was performed using ABI 7900HT Fast Real-Time PCR System using primers specific for (A) Olr1612; (B) Olr1671; (C) Olr322; (D) Olr735. \*p<0.05, n=4-5 rats per group by 2-tailed t-test.

225x170mm (300 x 300 DPI)

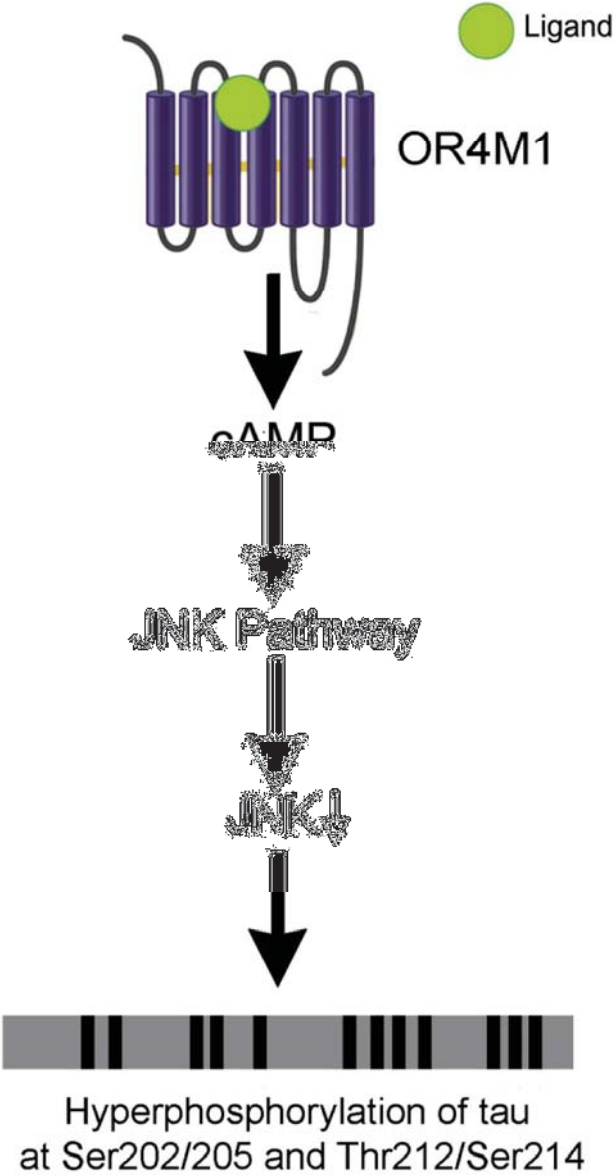


Figure VI. Scheme of working hypothesis.  
72x136mm (300 x 300 DPI)



Gene	Forward	Reverse
Olr322	AAACTACACCAGGGTCAAAGAA	CACCAGGAGAAGGAAGAGAAAC
Olr735	GACCCATCCAGCACATTGAT	GGTTGGTGAGAGGAGAATTGAG
Olr1612	GCCTGTGCCAATACTTTTCTG	CTTGAGTGTTTCTTGAGCATGG
Olr1671	GTGATGTCCTATGACCGCTATG	TGAAGTGCTGAGGTGGTAAAG
TBP	CACCAATGACTCCTATGACCC	CAAGTTTACAGCCAAGATTCACG

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Cluster	ZINC ID	cAMP (% of control) Mean ± STD
C1	00387281	72.37 ± 11.49
C2	12916861	81.38 ± 23.31
	32740605	113.81 ± 14.83
	09255703	115.59 ± 25.83
	9578142	88.90 ± 22.88
	13021362	89.33 ± 36.94
	09912561	63.98 ± 19.14
C3	69351464	65.20 ± 23.20
C4	12974343	71.82 ± 33.32
	05352464	92.27 ± 23.98
C5	14148934	107.37 ± 25.22
	14149349	119.59 ± 39.55
	10913993	134.95 ± 28.33
	10913792	102.15 ± 14.23
C6	03437366	70.56 ± 13.00
C7	12768603	62.32 ± 20.52
C8	71896059	47.75 ± 10.34
C9	05937266	90.38 ± 3.98
C10	71821329	49.72 ± 3.08
C11	05455334	62.70 ± 6.34
C12	65538929	40.86 ± 5.88
C13	08987000	61.39 ± 10.79
C14	30481138	82.90 ± 15.93
C15	10915775	261.89 ± 39.03*
C16	32777649	428.61 ± 46.10*